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Identification of a novel variant of the RET proto-oncogene in a novel family with Hirschsprung's disease

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Abstract

***Purpose*:** Hirschsprung's disease (HSCR) is a congenital disorder of the enteric nervous system characterized by the absence of ganglion cells in the Auerbach's and Meissner's plexuses. Although about 7% of cases are hereditary, the causal mutations have not been completely characterized. We encountered a novel family with inherited HSCR and screened them for causal mutations.

***Methods*:** A Japanese family of five female patients and six unaffected individuals was subjected to a whole-exome analysis with a next-generation sequencer.

***Results*:** After exome sequencing and the annotation of mutations, we identified co-segregated mutations with sequential filtering steps via a standard protocol. Eight mutations were identified: 2 on chromosome 10 and 6 on chromosome 11. We used pathogenicity prediction tools such as Genomic Evolutionary Rate Profiling, SIFT, and PolyPhen2 to predict the impact of mutations on the protein activity. S922Y, a novel mutation of *RET*, was identified as a likely causal mutation. In addition, a mutation of rs2435357T, known enhancer of *RET* located in intron 1 of *RET*, was detected in this family.

***Conclusion*:** The coexistence of *RET* mutations in both the exon (S922Y) and intron1 (rs2435357T) indicated a risk of HSCR in this family.

(191words/Limit200)

Keywords: Hirschsprung's disease, Familial, Short segment, *RET*, Genome wide association study

Introduction

Hirschsprung's disease (HSCR) is a congenital disorder of the enteric nervous system that is characterized by the complete absence of ganglion cells in the Auerbach's and Meissner's plexuses from continuous segments of the intestinal tract. This defect arises from a failure of the cranio-caudal migration, proliferation, differentiation or colonization of precursor enteric neural crest cells in the gastrointestinal tract. HSCR occurs in approximately 1 of 5,000 live births [1]. The incidence of familial HSCR is reported to range from 5%-20% [2,3]. HSCR can be classified into two major groups based on the length of the aganglionic lesion: patients with aganglionosis as far as the sigmoid colon (short segmental type, S-HSCR) and patients with aganglionosis beyond the sigmoid colon (long segmental type, L-HSCR). S-HSCR is male-dominant and sporadic [4], the proportion of familial cases is higher for L-HSCR than for S-HSCR. Several genes, such as *RET, ECE1, EDN3, EDNRB, GDNF, NRTN, SOX10, ZFHX1B, PHOX2B, TCF4,* and *KIAA1279*, have been found to be associated with the onset of HSCR [2]

RET was first identified and is the most commonly observed causality for HSCR. However, *RET* was originally identified as an oncogene activated by DNA rearrangement. The *RET* proto-oncogene located on chromosome 10q11.2 encodes a 1114-amino acid transmembrane receptor with a cadherin-related motif and a cysteine-rich domain in the extracellular domain [5].

RET mutations appeared to account for approximately 50% of familial L-HSCR and 10%-20% of sporadic cases (mostly S-HSCR) [6]. A variety of mutations have been identified by the direct sequencing of *RET*. However, genetic abnormalities cannot explain all cases of HSCR from current research reports, so further screening for additional HSCR-causing mutations is needed.

We experienced a novel family comprising five female patients with S-HSCR, and screened them in order to identify the causal mutations using an exome analysis.

Methods

Sample recruitment

We treated 109 HSCR patients from 1984 to 2016 and identified 6 familial cases (containing 16 of the treated patients [14%]) at our institution. All patients with HSCR were diagnosed by a rectal mucosal biopsy and underwent definitive surgery at our institution. We experienced one unique HSCR family with five female patients. The extent of aganglionosis in these five female patients was as far as the sigmoid colon (S-HSCR). In this family, three generations were recruited, and no males (including the fathers of the patients) were affected (Fig. 1). In light of the unique hereditary form of this disease, this family was screened for new mutations of causal genes of HSCR.

Fig. 1

We planned to perform an exome analysis using DNA that had been extracted from the peripheral blood in accordance with the standard protocol.

This study was conducted under the Ethical Guidelines for Human Genome/Gene Analysis Research and approved by The Research Ethics Committee of Kagoshima University Hospital (registration number; 275). All of the participants or their parents provided their informed consent to participate in this study.

Exome sequencing data analysis

Exome sequencing was performed using the SureSelect Human All Exon Kit® (Agilent Technologies, Santa Clara, CA, USA). The captured DNA libraries were subjected to high-throughput sequencing using the HiSeq2000® system (Illumina, San Diego, CA, USA), a next-generation sequencer.

The genome reading results were mapped to the reference genome (University California Santa Cruz [UCSC] Genome Browser, which provides human whole-genome sequences, https://genome.ucsc.edu/). We used the genome analysis software program

Burrows-Wheeler Aligner® (BWA) v.0.7.9. BWA-generated Sequence Alignment/Map (SAM) files were converted to a Binary Alignment Map (BAM) format to identify the gene and then sorted and indexed using SAM tools® v.0.1.18. Duplicated reads were marked with Picard® v.1.102 (https://github.com/broadinstitute/picard). The files obtained in BAM format were analyzed using The Genome Analysis Toolkit® (GATK) v.2.7 to annotate the gene. To perform gene annotation, we used the Reference Sequence gene database (UCSC). The annotation of normal variant and mutation was detected based on the single nucleotide polymorphism database (dbSNP) [7]. The frequency of mutations was obtained from the 1000 Genomes Project database, the result of international research of concerning human genetic variants [8].

Methodology for narrowing down the candidate mutations

The mutations detected through exome sequencing of the family's samples were further analyzed by performing four filtering steps based on different criteria.

We obtained the exonic or splicing mutations from exome sequencing data analysis as described in previous sections. We narrowed down the candidate mutations as follows: (1) Removal of the synonymous mutations that do not affect the amino acid expressions, leading non-synonymous or splicing site mutations. (2) Removal of the mutations recorded in the dbSNP and those with minor allele frequency (MAF) >1% in the 1000 Genomes Project database in order to obtain rare mutations as potential candidates, as HSCR is a rare disease. The mutations were also assessed in 1,208 Japanese individuals from the Human Genetic Variation database (HGVD) (http: //www. genome.med.kyoto-u.ac.jp/SnpDB/index.html). This process left only novel or rare mutations. (3) Removal of mutations with allele frequencies >0.7 or <0.3, leaving only heterozygous mutations. (4) Removal of mutations observed in unaffected members and the selection of common mutations among affected patients and genetic carriers. After performing these steps, we obtained the final candidate mutations list.

Sanger sequencing

We examined all of the family members by Sanger sequencing to confirm the finding. Sanger sequencing was also able to detect abnormalities in the intron area. Polymerase chain reaction (PCR) primers were designed using Prime3Plus (open source; http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/), and the target sequences were amplified by PCR and then sequenced using the Sanger method on a 3730 DNA Analyzer® (Thermo Fisher Scientific, Inc. Waltham, MA, USA).

Results

We performed the exome analysis for a family of five female patients and six unaffected individuals and attempted to identify the mutations shared by the affected patients. After mapping to the reference genome, approximately 7 GB of sequences per sample were obtained, representing 93% coverage of all exons with a depth of at least 20 reads; these data seemed to be sufficient for further analyses. After the annotation of the mutations, we performed sequential filtering in order to narrow down the candidate mutations.

Results of screening candidate SNPs and deletions

After narrowing down the candidate mutations, as shown in Fig.2, around 300 novel or rare heterozygous mutations were detected. Finally, we selected the variants showing the same mutations shared by patients (III:1, III:2, III:4, III:5, IV: 2) and unaffected gene carriers (II: 1 and II: 3). the mutations that were not shared by unaffected individuals were excluded. IV: 1 was excluded from the co-segregation analysis, because he was not determined a gene carrier. Ultimately, eight SNPs were identified: two mutations on chromosome 10 and six mutations on chromosome 11 (Fig. 2).

Fig.2

Results of candidate mutations and predicting the effects of amino acid substitutions

In order to select the candidate mutations, we use three predicting tools that reflect the protein function. For the annotation of mutation conservation across species, we used the Genomic Evolutionary Rate Profiling (GERP) score [9]. The higher the GERP score, the more important the mutation. Four mutations showed relatively high conservation scores (>4.0) (Table 1). We also used the pathogenicity prediction tools SIFT [10] and PolyPhen2 [11] to predict the impact of mutations on the protein activity. A lower SIFT score and higher PolyPhen2 score are associated with a decrease in the protein activity. The SIFT score ranges from 0 to 0.17, PolyPhen2 ranges from 0 to 1. Given these findings, the mutations of *RET* and *OR5AR1* are the most likely deleterious candidates. However, *OR5AR1* encodes an olfactory receptor and is not related to intestinal embryogenesis. Conclusively, a mutation in codon c.C2765A (p. S922Y) of *RET* was the most probable causal mutation in this family (Fig.2). Because HSCR is a multifactorial disorder, *RET* along with other candidate genes together may play an interactive role in the pathogenesis.

Table 1

Fig.2

Sanger sequencing confirmation and co-segregation

We also performed Sanger sequencing to confirm the *RET* status for all of the family members. S922Y located in an Exon of *RET* and an SNP (rs2435357T) located intron 1 of *RET* were detected. S922Y of *RET* showed consistent mutations in the affected patients of III:1, III:2, III:4, III:5, IV:2 and in the carriers of II:1, II:3 and IV:1. This pattern could not be explained by a single mutation, in S922Y of *RET*, so we further examined other common risk mutations. Rs2435357T of *RET*, which is known to be a *RET* enhancer, showed consistent mutations in the affected patients and in the gene carriers II: 1, II: 3 and IV: 1 as shown in Fig.3.

Fig.3

Altogether, we identified two mutations of *RET* in this family. The Coexistence of *RET*

mutations, both at the exon (S922Y) and intron1 (rs2435357T) indicated a risk of HSCR in this family.

Discussion

HSCR, the most common hereditary intestinal obstruction, shows considerable variation and complex inheritance patterns. Coding sequence variants in *RET, ECE1, EDN3, EDNRB, GDNF, NRTN, SOX10, ZFHX1B, PHOX2B, TCF4,* and *KIAA1279* lead to L-HSCR and syndromic HSCR, such as Waadenburg syndrome [2], but fail to explain the onset of common S-HSCR.

In this study, we performed exome analyses to identify the causal mutations of HSCR in a 3-generation Japanese family comprising 11 members (5 female patients and 6 unaffected individuals). After exome sequencing and several filtering steps, two mutations on chromosome 10 and six mutations on chromosome 11 were detected in this family. The major findings of this study are as follows: (1) S922Y as a mutation of exon of RET was first detected in the patients with HSCR. (2) rs2435357T, which is an enhancer of *RET* and located in intron1, was also detected in the patients with HSCR.

Eight candidate genes were detected in the patients with HSCR and carriers from this family. On chromosome 10, *RET* is a proto-oncogene and well-recognized to be involved in HSCR. *A1CF* encodes the *APOBEC1* complementation factor involved in RNA editing or RNA processing events. On chromosome 11, *PRDM11* encodes a putative novel tumor suppressor, which controls the expression of key oncogenes. *F2* encodes coagulation factor II, which is proteolytically cleaved to form thrombin in the coagulation cascade and plays a key role in maintaining vascular integrity during development and postnatal life. *OR5AR1* encodes an olfactory receptor. *SLC22A12* encodes a member of the organic anion transporter (OAT) family and acts as a urate transporter to regulate the urate levels in blood. *ADRBK1* encodes a G

protein-coupled receptor kinase 2. Abnormal coupling of beta-adrenergic receptor to G protein is involved in the pathogenesis of heart failure. *XRRA1* encodes X-ray radiation resistance-associated protein 1. However, additional evidence is needed to understand the whole picture of the pathogenesis.

A mutation of *RET* was deemed the most likely causal mutation among the eight mutations, according to pathogenicity prediction tools. In particular, S922Y of *RET*, which was detected in this study, had not been previously reported to be associated with HSCR. Position 922 of *RET* located tyrosine kinase domain. According to an *in silico* analysis, the S922Y mutation in the protein kinase domain increases the size and hydrophobicity of the protein, whereas the charge remains unchanged, and the polarity is decreased [12].

Based on a functional evaluation of *RET* with HSCR mutations, at least four mechanisms of RET dysfunction are responsible for the development of HSCR. First, most mutations in the RET extracellular domain were found to impair the receptor activity to GDNF, which results in deficient ganglion cells in the lower digestive tract during embryogenesis. Second, a couple of mutations were found to impair some proteins binding to *RET*, resulting in a *RET*-mediated signaling defect. Third, several mutations in the kinase domain almost abolished *RET* tyrosine kinase activity; these mutations occurred at highly conserved regions among members of the tyrosine kinase family. Fourth, several mutations that were also identified in the kinase domain severely impaired the activation of phospholipase Cγ [13]. S922Y was located at the tyrosine kinase domain and was able to impair the function of protein. These major changes presumably contribute to cause protein dysfunction and lead to HSCR in this family.

In *Ret* deficient mice, the aganglionosis phenotype exhibits full penetrance and is associated with a kidney deficit [14]. In contrast, *RET* variant-associated HSCR is usually characterized by incomplete penetrance. Uesaka et al. [15] found that the low expression of *Ret*

in mice caused apoptosis of enteric neurons in the colon. This phenomenon led to enteric neuronal cell death related to the onset of HSCR. They found that the aganglionosis phenotype emerged in mice when the *Ret* expression was <30%, suggesting the importance of the *RET* dosage in the development of HSCR. Kitamura et al. reported that a combination heterozygote of S922Y and M918T mutations was found in a MEN2B patient. However, three other family members with only S922Y were asymptomatic [16] . These results indicate that S922Y is functional but not sufficient to cause a disease phenotype.

Recent genetic studies have reported a common variant in a potential enhancer region of *RET* that is strongly associated with HSCR. Emison et al. showed that a common non-coding *RET* mutation (rs2435357T) as a *RET* enhancer of intron 1 is strongly associated with HSCR susceptibility [17]. rs2435357T is known to be a strong genetic factor, particularly for typical HSCR (male, S-HSCR, sporadic and non-syndromic cases) [18].

However, in this family, all affected patients and carriers had rs2435357T on chromosome 10 with S922Y mutations. In addition, the affected members were all female, had S-HSCR, and simplex cases contrasting with Emison's findings. Approximately 25% of Japanese have an rs2435357T mutation according to the 1000 genome project in Japan [8]. However, HSCR occurs in approximately 1 of 5,000 live births, the rs2435357T mutation alone cannot explain the onset of HSCR. Emison et al. also noted that two- or multiple-hits are needed in the pathogenesis of HSCR [19]. Given these present and previous findings, both the S922Y and rs2435357T mutations of *RET* may have played an interactive role in onset of HSCR in this family.

In conclusion, we performed exome sequencing for a novel family with HSCR to identify the genetic causality. We detected the S922Y mutations and rs2435357T mutations of *RET* in this study, but these mutations are not sufficient to explain the pathogenesis. The co-existence and interaction of two *RET* mutations is a key finding in this unique familial series

of HSCR, and further studies should perform functional assessments using knockout models of these genetic mutations.

Author Disclosure Statement

No competing financial interests exist.

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Legends for Figure/Table

Figure 1 **Family tree of the familial patients**

The filled circles denote affected individuals and the open symbols represent unaffected subjects. All affected patients were female and had short segment aganglionosis. II:1 and II:3 are probably genetic carriers. IV:1 was unaffected and genetic background was not determined.

Figure 2 **The results of screening candidate mutations**

The upper table shows the results of the number of the candidates left after the narrowing down the list, as shown in Figure 2.

The lower table shows the chromosome and position of eight mutations and the name of the candidate genes.

Figure 3 **RET S922Y and SNP of rs2435357 status in this family**

This figure shows the variants of S922Y and rs2435357 in this family.

The filled circles denote affected individuals, and the filled squares denote carriers.

The open symbols represent unaffected subjects.

All of the affected members and carriers have mutations in both *RET* S922 and rs2435357T.

The red character is a mutation of the allele.

RET S922Y: Its original allele is C,

All affected members have allele A.

rs2435357: SNP name in the intron 1 of *RET* on chromosome 10.

Its original allele is C.

S-HSCR: Short-segment aganglionosis of Hirschsprung's disease.

Table 1 Candidate mutations and the evaluation score of the predicting tools

GERP: Genomic Evolutionary Rate Profiling score.

The higher the GERP score, the more the important role of a mutation.

SIFT: Sorting Intolerant Form Tolerant score.

A lower SIFT score is associated with a decrease in the protein activity.

PolyPhen2: Polymorphism Phenotyping v2 score.

A higher PolyPhen2 score is associated with a decrease in the protein activity.

Figure 1: Family tree of the familial patients

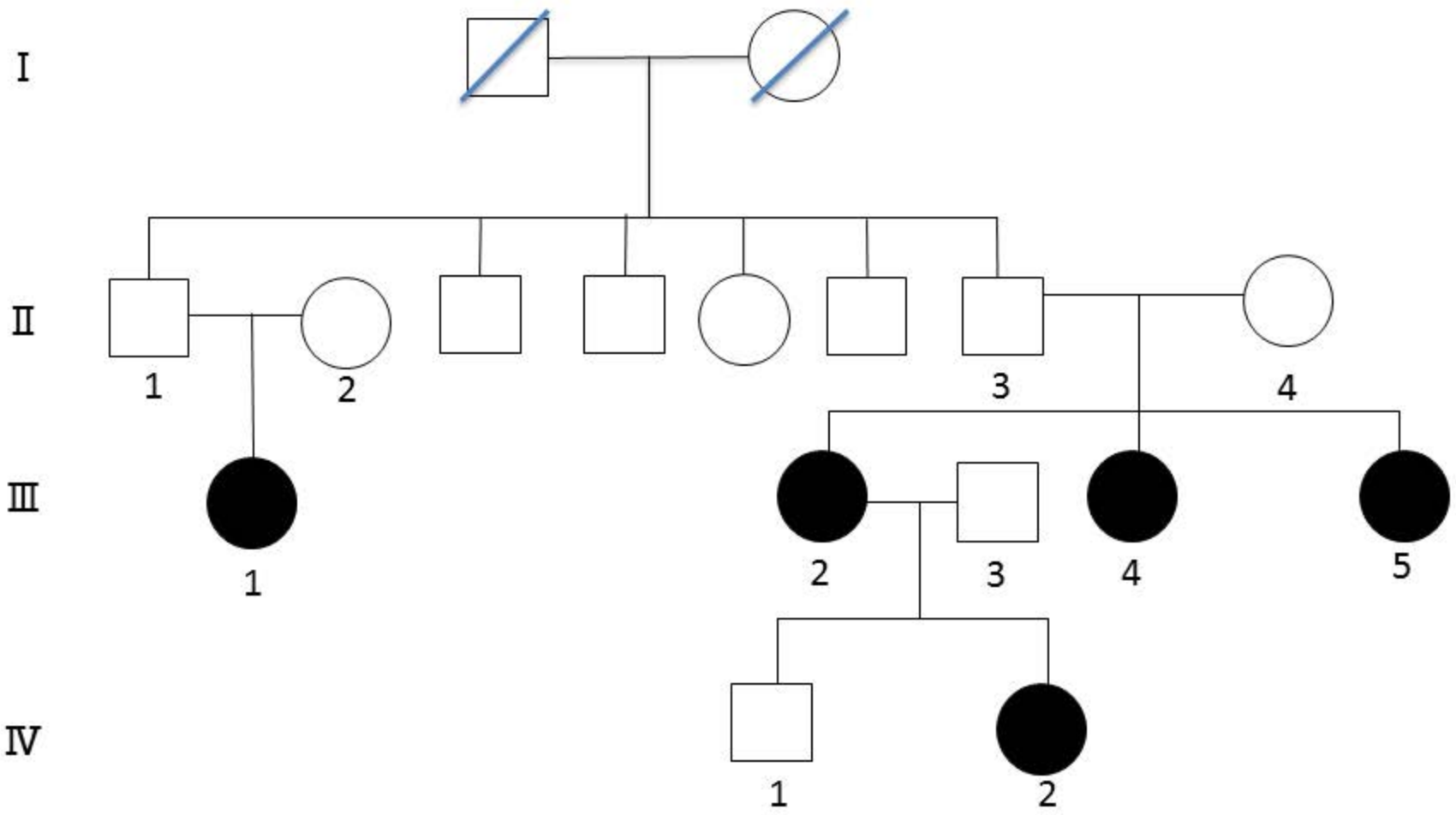

Figure 2: The results of screening candidate mutations

	Ⅱ:1	Ⅱ:2	Ⅱ:3	Ⅱ:4	Ⅲ:1	Ⅲ:2	Ⅲ:3	Ⅲ:4	Ⅲ:5	Ⅳ:1	Ⅳ:2
Status	unaffected carrier	unaffected	unaffected carrier	unaffected	affected	affected	unaffected	affected	affected	unaffected informative	affected
Exonic/splicing	20,845	20,840	20,838	20,919	20,599	20,710	20,931	20,845	20,922	20,879	20,837
Nonsynonymous /splicing/stop	10,599	10,522	10,580	10,701	10,327	10,582	10,563	10,599	10,683	10,553	10,544
Unrecorded in dbSNP	1,723	1,585	1,615	1,781	1,586	1,720	1,617	1,723	1,723	1,652	1,679
1000 genomes < 1%	1,660	1,511	1,556	1,716	1,522	1,666	1,534	1,660	1,660	1,581	1,613
Heterozygous 0.3 -0.7	324	305	352	356	287	320	323	324	375	334	363

Ⅳ:1 was excluded for filtering to narrow down the candidate mutaions

8 common mutations among affected and genetic carriers

Chromosome	Position	Reference allele	Observed allele	Gene name	Mutation	Amino Acid Change
10	43617428	C	A	RET	c.C2765A	p.S922Y
10	52573747	C	T	A1CF	c.C1193T	p.G398D
11	45203382	T	C	PRDM11	c.T65C	p.V22A
11	46747457	G	A	F2	c.G608A	p.S203N
11	56431339	T	C	OR5AR1	c.T178C	p.Y60H
11	64366349	C	T	SLC22A12	c.C1024T	p.R342C
11	67047365	T	C	ADRBK1	c.T497C	p.I166T
11	74554918	G	C	XRRA1	c.G1281C	p.N427K

Figure 3: **RET S922Y and SNP of rs2435357 status in this family**

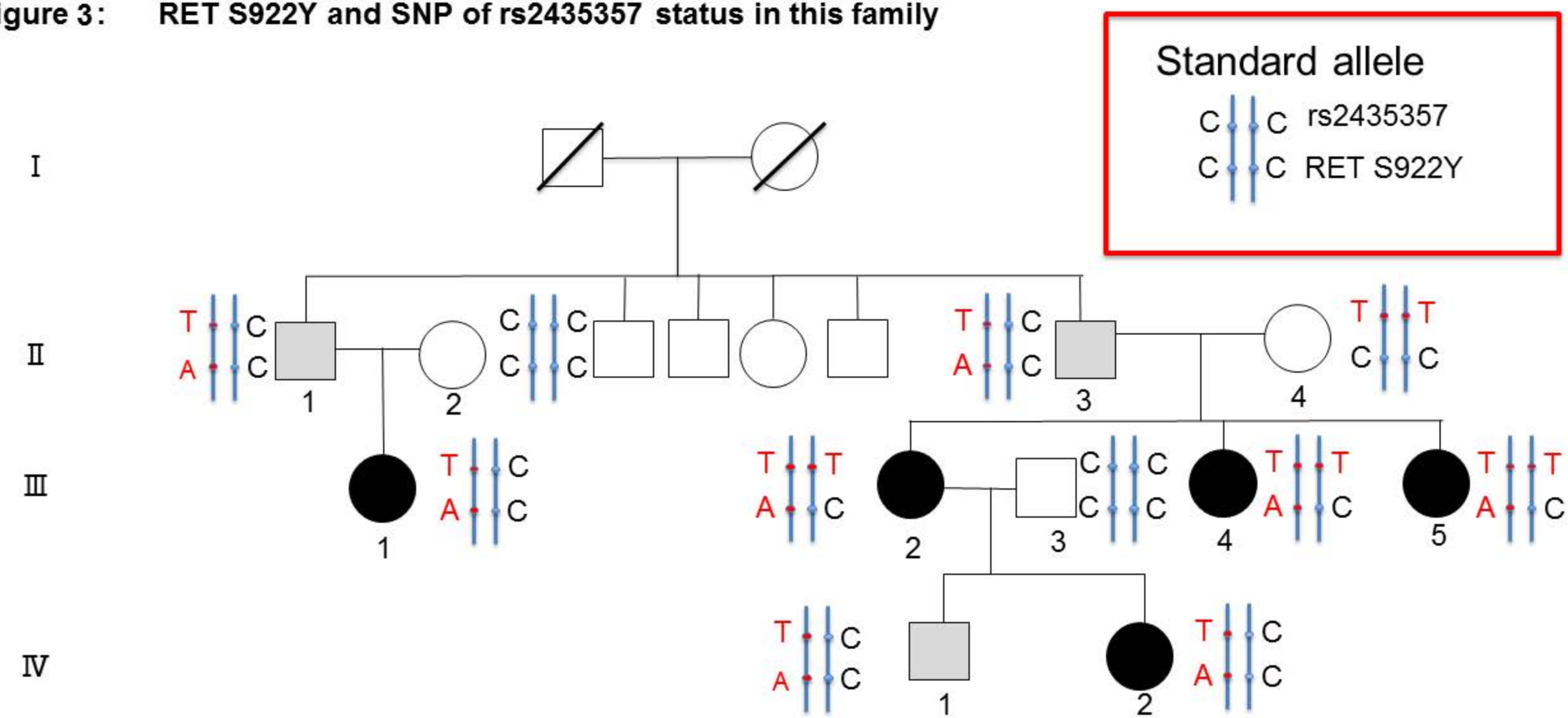

Table 1 **Candidate mutations and the evaluation score of the predicting tools**

Chromosome	Position of mutation	original allele	changed allele	Gene	Pathogenicity predicting tools		
					GERP	SIFT	Polyphen2
10	43617428	C	A	*RET*	**5.43**	**0**	**1**
10	52573747	C	T	*A1CF*	**5.87**	0.12	**0.984**
11	45203382	T	C	*PRDM11*	2.78	**0**	0.954
11	46747457	G	A	*F2*	1.47	**0**	0.001
11	56431339	T	C	*OR5AR1*	**5.25**	**0**	**1**
11	64366349	C	T	*SLC22A12*	-1.39	0.07	0.096
11	67047365	T	C	*ADRBK1*	**5.13**	0.11	0.199
11	74554918	G	C	*XRRA1*	-2.36	0.17	**0.993**